

EXPERIMENTAL STUDIES
IN
NUCLEAR MAGNETIC RELAXATION
AND
MOLECULAR DIFFUSION

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This review summarizes results from the following papers
which will be denoted by reference numbers 1 - 9 :

1. J. Andrasko and S. Forsén:

"Pulsed NMR Studies on Na^+ Binding to Simple Carbohydrates".
Biochem. Biophys. Res. Commun. 52, 233 (1973).

2. T.E. Bull, J. Andrasko, E. Chiancone and S. Forsén:

"Pulsed Nuclear Magnetic Resonance Studies on ^{23}Na , ^7Li
and ^{35}Cl Binding to Human Oxy- and Carbon Monoxyhaemo-
globin".

J. Mol. Biol. 73, 251 (1973).

3. J. Andrasko, I. Lindquist and T.E. Bull:

"Quadrupole Relaxation in Solution of Humic Acid".
Chem. Scr. 2, 93 (1972).

4. J. Andrasko:

"Nonexponential Relaxation of $^{23}\text{Na}^+$ in Agarose Gels".
J. Magn. Res. 16, 502 (1974).

5. J. Andrasko and S. Forsén:

"Deuterium Magnetic Relaxation Study of Molecular Dyna-
mics. The Interactions of EDTA with Some Metal-free
Proteins".

Chem. Scr. 6, 163 (1974).

6. J. Andrasko:

"Water in Agarose Gels Studied by NMR Relaxation in the Rotating Frame".

Biophys. J. In press.

7. J. Andrasko and S. Forsén:

"NMR Study of Rapid Water Diffusion Across Lipid Bilayers in Dipalmitoyl Lecithin Vesicles".

Biochem. Biophys. Res. Commun. 60, 813 (1974).

8. J. Andrasko:

"Water Diffusion Permeability of Human Erythrocytes Studied by a Pulsed Gradient NMR Technique".

Biochim. Biophys. Acta. In press.

9. J. Andrasko:

"Measurement of Membrane Permeability to Slowly Penetrating Molecules by a Pulsed Gradient Method".

J. Magn. Res. In press.

These articles concern NMR investigations of biochemical and biological systems and can thematically be divided into two different topics:

- a) NMR relaxation studies of interactions between small molecules or ions and macromolecules or other molecules of biological interest.

Most attention has been given to investigations by quadrupole relaxation (papers 1-5), and dipolar relaxation has been studied in paper 6.

- b) Measurement of diffusion permeability of biological and model membranes to ions and neutral molecules.

In paper 7, the permeability of dipalmitoyl lecithin vesicles to water has been studied using NMR relaxation methods. Papers 8-9 deal with the possibilities of NMR diffusion techniques for the measurement of membrane permeabilities to slowly (9) and rapidly (8) penetrating species.

I. Interactions of ions and small molecules with macromolecules.

Quadrupole relaxation.

For nuclei with spin quantum numbers greater than 1/2 the dominant relaxation mechanism is usually given by the interaction between the electric field gradient and the nuclear electric quadrupole moment. Some typical quadrupolar nuclei which appear in biological systems are $^{23}\text{Na}^+$, $^{35}\text{Cl}^-$, $^{39}\text{K}^+$, etc. The fluctuations of the electric field gradient are usually characterized by a correlation time τ_c , which in liquids may be rotational (in molecules such as CCl_4) or diffusional (in solvated monoatomic ions). Under extreme narrowing conditions, when the product of the nuclear Larmor frequency ω_0 and the correlation time τ_c is much less than 1, the relaxation times T_1 and T_2 are equal and given by¹

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{3}{40} \frac{2I + 3}{I^2(2I-1)} \left(\frac{e^2 Q q}{h} \right)^2 \tau_c = K \nu_Q^2 \tau_c \quad [1]$$

where eq is the electric field gradient to which the electric quadrupole moment of the nucleus (eQ) is coupled and I is the nuclear spin quantum number. K is a constant depending on I and $\nu_Q \left(= \frac{e^2 Q q}{h} \right)$ is the quadrupole coupling constant. In Eq. 1 any deviation of the electric field gradient from axial symmetry is neglected. For a given quadrupolar nucleus, the relaxation times are primarily determined by the electric field gradient and by the correlation time.

In studies of the binding of ions to macromolecules the correlation time describing the motion of the bound ion is often several orders of magnitude longer than the value for the "free" ion so that the conditions of extreme narrowing may not be satisfied. For a nucleus with $I=1$ (^2H , ^{14}N etc.) the relaxation times are given by¹

$$\begin{aligned} \frac{1}{T_1} &= K \nu_Q^2 \left(\frac{\tau_c}{1 + \omega_o^2 \tau_c^2} + \frac{4 \tau_c}{1 + 4 \omega_o^2 \tau_c^2} \right) \\ \frac{1}{T_2} &= K \nu_Q^2 \left(1.5 \tau_c + \frac{2.5 \tau_c}{1 + \omega_o^2 \tau_c^2} + \frac{\tau_c}{1 + 4 \omega_o^2 \tau_c^2} \right) \end{aligned} \quad [2]$$

It follows from Eq. 2 that the dependences of T_1 and T_2 on τ_c are of different character; T_1 exhibits a minimum when $\omega_o \tau_c \approx 0.62$. Furthermore, for a given τ_c , T_1 and T_2 are functions of the Larmor frequency ω_o . This fact can be used to determine the value of τ_c experimentally.

For nuclei with spin quantum number greater than 1, when the approximation $\omega_o \tau_c \ll 1$ is not valid, the relaxation times T_1 and T_2 are no longer defined and the relaxation is non-exponential². Thus for spin-3/2 nuclei (^{23}Na , ^{35}Cl , ^7Li , ^{79}Br ...), which represent ions frequently studied in biochemical systems, the longitudinal and transverse relaxation are generally the sums of two exponentials. The longitudinal relaxation consists of a faster exponential process to 20% (time constant $1/T_1^*$) and of a slower exponential process to 80% (time constant $1/T_1^{**}$)^{2,40}

$$\begin{aligned} \frac{1}{T_1^*} &= \frac{1}{10} \nu_Q^2 \left(\frac{\tau_c}{1 + 4 \omega_o^2 \tau_c^2} \right) \\ \frac{1}{T_1^{**}} &= \frac{1}{10} \nu_Q^2 \left(\frac{\tau_c}{1 + \omega_o^2 \tau_c^2} \right) \end{aligned} \quad [3]$$

In the transverse relaxation the contributions of faster ($1/T_2^*$) and slower ($1/T_2^{**}$) components are 60% and 40%, respectively².

$$\begin{aligned} \frac{1}{T_2^*} &= \frac{1}{20} \nu_Q^2 \left(\tau_C + \frac{\tau_C}{1 + \omega_O^2 \tau_C^2} \right) \\ \frac{1}{T_2^{**}} &= \frac{1}{20} \nu_Q^2 \left(\frac{\tau_C}{1 + \omega_O^2 \tau_C^2} + \frac{\tau_C}{1 + 4\omega_O^2 \tau_C^2} \right) \end{aligned} \quad [4]$$

Effect of chemical exchange.

Chemical exchange can influence NMR relaxation times in a number of ways depending on the rate of exchange³. One special case of chemical exchange will be mentioned here. For chemical exchange between an ion (or small molecule) solvated in water and an ion attached to a biological macromolecule at one or more binding sites, the relaxation times of the "free" ion are usually much longer than those of the bound ion. Furthermore, the concentration of macromolecules in solution is generally quite small and the fraction of bound ions is much lower than the fraction of "free" ions. Then, when a nucleus undergoes chemical exchange between sites A and B, where site A is ascribed to the "free" nucleus and site B to the bound nucleus, the observed relaxation times are given by^{4,5,41}

$$\begin{aligned} \frac{1}{T_1} &= \frac{1}{T_{1A}} + \frac{P_B}{P_A} \frac{1}{T_{1B} + \tau_B} \\ \frac{1}{T_2} &= \frac{1}{T_{2A}} + \frac{P_B}{P_A} \frac{1}{T_{2B} + \tau_B} \end{aligned} \quad [5]$$

where P_A and P_B are the fractions of nuclei at site A and B and τ_B is the mean lifetime of nuclei at site B. In the expression for T_2 the chemical shift difference between the nucleus at site

A and B is assumed to be much smaller than $1/T_{2B}$.

T_{1A} and T_{2A} are values measurable for samples in the absence of the macromolecule so that the second terms in Eq. 5 which contain the information about the interaction studied can be directly obtained from the observed relaxation times.

The above expressions may easily be modified for the presence of more than one set of binding sites if the summation over these different sites is introduced. For very rapid exchange among m sites, when the lifetimes of nuclei at each of these sites are much shorter than the corresponding relaxation times, the observed relaxation rates are a weighted average of the relaxation rates at the m available sites; i.e.,

$$\frac{1}{T_i} = \sum_{j=1}^m \frac{P_j}{T_{ij}} \quad ; \quad i = 1, 2 \quad [6]$$

where P_j is the probability of finding the nucleus at site j and T_{ij} is the relaxation time of the nucleus at site j .

Analysis of binding of quadrupolar ions.

NMR relaxation measurements may give several different pieces of information about the binding process:

- a) the fractions of bound molecules or ions and the corresponding binding constants;
- b) dynamics of bound species in terms of the correlation times;
- c) the mean lifetime τ_B which describes the kinetic of the exchange process;
- d) the quadrupole coupling constant ν_Q for ions bound to macromolecules. The value of this coupling constant is dependent on the character of the binding. Large ν_Q 's are typical for covalent binding, while ionic binding

is characterized by smaller ν_Q values.

The sensitivity of the relaxation times of a nucleus with quadrupole moment to its environment is a basic principle of work with "halide probes"⁶. Another example of the use of this sensitivity for a quadrupolar alkali cation for investigations of weak complexes in solution is shown in paper 1. This paper deals with complex formation between monosaccharides and sodium ions in aqueous solutions. Sugars possess several hydroxy groups which might be able to bind metal cations. Complexes between neutral sugars and metal cations were studied by proton NMR, and it was found that certain configurations of hydroxy groups in sugar molecules favour complex formation⁷. It appeared that sugars containing an ax-eq-ax sequence of three hydroxy groups in a six-membered ring, or a cis-cis sequence in a five-membered ring, form complexes with metal ions, particularly with alkaline-earth metal ions. This hypothesis has been confirmed in paper 1 by measurements of the ²³Na relaxation for aqueous NaCl in the presence of different monosaccharides. Only a few of the carbohydrates investigated caused recognisable changes in the sodium relaxation rate, thus indicating complex formation. These monosaccharides had the required arrangement of hydroxy groups in their most stable conformations. The measurement of the ²³Na relaxation was found to be sensitive to the presence of very weak complexes, and observable changes in sodium relaxation rate were noticed even for sugars with the favourable sequence of hydroxy groups in less stable conformations. The ability of monosaccharides to form complexes with Na⁺ was also found to be independent of pH between pH = 2 and 10.

Attempt to investigate the dynamics of bound spin-3/2 nuclei and to obtain some quantitative information about the binding

process have been made in papers 2 and 3. In both studies the macromolecular tumbling motion was sufficiently slow to produce non-extreme narrowing conditions for bound ions. According to Eqs. 3 and 4 the observed longitudinal and transverse relaxation should be non-exponential under these conditions, in virtual disagreement with experimental observations in papers 2 and 3. Bull⁸ investigated the effect of chemical exchange between two sites on the quadrupole relaxation of spin-3/2 nuclei. For the case where the relaxation appears to be exponential although the nuclei at one of the sites are under non-extreme narrowing conditions, an approximate expression for the relaxation times was found. With the same assumptions as those in Eq. 5, the observed T_1 and T_2 are given by⁸

$$\begin{aligned} \frac{1}{T_1} &= \frac{1}{T_{1A}} + \frac{P_B}{P_A} \left(\frac{0.2}{T_{1B}^* + \tau_B} + \frac{0.8}{T_{1B}^{**} + \tau_B} \right) \\ \frac{1}{T_2} &= \frac{1}{T_{2A}} + \frac{P_B}{P_A} \left(\frac{0.6}{T_{2B}^* + \tau_B} + \frac{0.4}{T_{2B}^{**} + \tau_B} \right) \end{aligned} \quad [7]$$

Using the expressions above, the interactions between Cl^- , Na^+ , Li^+ and human haemoglobin were studied in paper 2. No evidence for Na^+ binding was found, and Li^+ showed only a slight tendency toward binding. The binding of Cl^- to oxyhaemoglobin was found to be strong, and this interaction was investigated more thoroughly by measuring the dependence of T_1 and T_2 for ^{35}Cl on temperature, Larmor frequency and concentration of added haemoglobin. The frequency dependence of the ^{35}Cl relaxation rates was followed over a limited frequency range at different temperatures in an attempt to separate the effects of correlation time (τ_C) and exchange time (τ_B) on the relaxation. An upper limit of 10^{-6} sec was obtained for the mean lifetime of

a chloride ion bound to the protein. The lower limit for τ_B was calculated to be about 10^{-8} sec according to the following equation^{9,42,43}:

$$\frac{1}{\tau_C} = \frac{1}{\tau_r} + \frac{1}{\tau_B} \quad [8]$$

where τ_r is the re-orientational correlation time. This equation shows that τ_B can not be shorter than the correlation time τ_C .

Another parameter calculated from the frequency dependence of the relaxation times was the product of the quadrupole coupling constant for the bound Cl^- and the square root of the number of binding sites per haemoglobin molecule, $\nu_Q \sqrt{N}$. Since N is a unknown parameter, the value of ν_Q could not be obtained.

Paper 3 is a similar study of the interactions between sodium ions and humic acid, an extract from peat soil¹⁰. The longitudinal relaxation of ^{23}Na in an aqueous solution of humic acid was exponential and the measurements were analysed using Eq. 7 in the same manner as in paper 2. Humic acid shows a great tendency to bind metal cations. At pH = 10 the presence of 0.09 weight % humic acid in 0.1 M NaCl solution caused an increase in the observed $1/T_1$ for ^{23}Na by a factor of 3.5. The binding capacity of humic acid was found to increase with pH and the exchange between bound and "free" sodium ions was very rapid. The correlation time for bound Na^+ was calculated to be ca. 2×10^{-9} sec at 28°C from the frequency dependence of T_1 . This value also serves as a lower limit for the mean lifetime of bound Na^+ .

The use of Eq. 7 in analysing the relaxation of spin-3/2 nuclei involved in chemical exchange is limited. This relax-

ation is generally non-exponential although the deviations of magnetization decays from exponentiality are difficult to observe, particularly when the concentration of the nuclei being investigated is low. The observation of non-exponential transverse relaxation for ^{23}Na in frog striated muscle has been reported¹¹. Since the sodium longitudinal relaxation was found to be exponential, such observations might also be interpreted in terms of the presence of slow exchange of Na^+ between two different environments.

A study of non-exponential relaxation of spin-3/2 nuclei was performed in paper 4 for Na^+ in agarose gels. Agarose is a polysaccharide with a large number of hydroxy groups, and it forms self-supporting gels in water. The gels were found to bind sodium ions. The observed transverse relaxation of ^{23}Na was clearly non-exponential, composed of 60% from one faster decay and of 40% from one slower decay in accordance with theory^{2,8}. Unlike the transverse relaxation which was non-exponential even in 1% agarose gels, the deviations of the ^{23}Na longitudinal relaxation from exponential behavior became apparent only for samples with high agarose contents. Since the longitudinal relaxation for bound Na^+ is composed of only 20% from one faster decay and of 80% from one slower decay this observation is easily explained. The composition of biological systems is usually unalterable. Thus, it can be difficult to extract the faster decay in the longitudinal relaxation, particularly for systems in which the observed relaxation of the quadrupolar nucleus is not much faster than the corresponding relaxation of the "free" nucleus.

Molecular dynamics of bound small molecules.

A detailed analysis of the relaxation of spin-3/2 nuclei

requires very good accuracy in the measurement of magnetization decays. In contrast, the relaxation times of nuclei with spin quantum number 1 are well defined even in the non-extreme narrowing case (Eq. 2). For studies of interactions between small molecules and macromolecules it should therefore be advantageous to label the molecule with deuterium and follow the interaction by means of changes in the deuterium relaxation rate.

In paper 5 the binding of ethylene diamine tetraacetic acid (EDTA) to some metal-free proteins was investigated. The interaction between EDTA and proteins was considered of interest in view of the frequent use of EDTA in biochemical preparations, in particular the preparation of metal-free apoenzymes. EDTA was labelled with deuterium on eight magnetically equivalent positions. Deuterium magnetic relaxation is particularly suited for the determination of various molecular parameters characterizing the EDTA - protein interaction, since the ^2H quadrupole coupling constant in the $\text{C}-^2\text{H}$ bond is presumably not altered by the binding and is nearly constant for most organic molecules. The proteins investigated were found to bind EDTA to an extent increasing with decreasing pH; the binding appeared to be weak and could be prevented by the addition of detergents such as sodium octylsulfate. Also the dynamics of EDTA molecules bound to bovine serum albumin and to γ -globulin was investigated. In both systems the exchange rate between bound and free EDTA was rapid compared to the corresponding relaxation rates. Thus, neglecting τ_B in Eq. 5, the correlation time τ_C for the bound molecule was calculated in two different ways:

- From the frequency dependence of T_1 .
- From the ratio T_{1B}/T_{2B} at one resonance frequency.

A significant discrepancy was noted on comparison of the results

obtained using the above procedures (a factor of 3 in τ_c). This indicates that the motion of bound EDTA can not be described by a single value of the correlation time. Therefore, the ^2H relaxation measurements were analysed assuming the presence of internal motion at site B. The effect of internal rotation on angular correlation functions for a magnetic nucleus attached to a macromolecule has been treated in the literature¹². Using diffusional rotation around one axis as a model for the internal motion, the dynamics of bound EDTA molecules was investigated. The calculation of the correlation times for macromolecular tumbling motion gave values which were in good agreement with the estimations obtained using the Stokes - Einstein formulae¹ in one of the two systems studied.

The analysis performed in paper 5 reveals that the presence of fast internal motion significantly influences the quadrupole relaxation times as well as their frequency dependence. The neglect of the effect of such motion and the use of Eq. 2 or Eqs. 3 and 4 for the relaxation times for bound nuclei will lead to incorrect values of the calculated binding parameters. Internal motions might frequently be present, particularly for small molecules attached to a macromolecule. The molecule may bind to the macromolecule in parts of the molecule which have some internal freedom of motion; furthermore, some rotation around one or more axis may occur within the molecule itself. Under these circumstances a quantitative analysis of the binding process from NMR relaxation measurements is difficult to carry out. The labeling of a molecule with deuterium on different positions might be fruitful in this analysis.

II. The state of water in biological systems.

The state of water in biological systems and the composition of the intracellular fluids are of great physiological interest. In recent years, NMR has been used extensively to study the organization and motion of water molecules in biological and model systems. NMR investigations of protein-water solutions, biopolymer-water mixtures and tissues have been reported. Early studies^{14,15} showed that water in muscles has restricted motional freedom compared with ordinary water. The reduced T_1 , and particularly T_2 , values for water protons indicated that the rotational and translational mobility of muscle water was nearly two orders of magnitude lower than ordinary liquid water. However, subsequent measurements of the diffusion coefficient showed that the average translational mobility of most muscle water is only reduced by a factor of ca. two^{13,16}. Thus, the NMR results could not be explained by assuming that water in biological systems is a homogeneous liquid with the molecular motion characterized by a single correlation time. A model of fast two-site exchange between a majority of "free" and a minor fraction of bound or hydrated water was introduced. The frequency dependence of proton^{17,18} and deuteron¹⁷ relaxation times for water in tissues and gels showed, however, that this simple model is inadequate. Today, NMR observations on the state of water in biological systems may be divided into two groups:

- a) A majority of investigations, particularly those on concentrated protein solutions, indicates that water in such systems exchanges rapidly among several states characterized by different correlation times. Using different models for the distribution of correlation times it has been found that the log-normal distribution is consistent with the frequency

and temperature dependence of T_1 for water protons in gels and muscles^{18,19}. In this model, a few percent of water has a distribution of mobilities between those of ice and liquid water and exchanges rapidly with the remaining fraction which is attributed to water in the normal state. The log-normal distribution of τ_c was subsequently applied to water motion in frozen systems such as frozen agarose gels²⁰.

- b) Several studies of water in skeletal muscle revealed deviations of the ^1H transverse relaxation from exponentiality^{21,22}. This was explained as due to the presence of at least three different fractions of non- (or slowly) exchanging water in the muscle. The slowly exchanging water associated with the intracellular macromolecules was found to be approximately 8-10% of the total tissue water²¹. This type of water was suggested to be non-freezable at temperatures below -10°C ²² and identical with the osmotically inactive water.

NMR measurements on water in biological systems are primarily concerned with investigations of the ^1H relaxation times T_1 and T_2 and their frequency dependence. In liquid water and ice, NMR relaxation occurs via dipolar interactions, mainly intramolecular, between water protons. The relaxation rates caused by this interaction are given by the following expressions (ref. 1, p. 290-294):

$$\begin{aligned} \frac{1}{T_1} &= C \left(\frac{\tau_c}{1 + \omega_o^2 \tau_c^2} + \frac{4 \tau_c}{1 + 4 \omega_o^2 \tau_c^2} \right) \\ \frac{1}{T_2} &= C \left(1.5 \tau_c + \frac{2.5 \tau_c}{1 + \omega_o^2 \tau_c^2} + \frac{\tau_c}{1 + 4 \omega_o^2 \tau_c^2} \right) \end{aligned} \quad [9]$$

where C is the interaction constant. For a case of rapid exchange of water between different motional states (Eq. 6) T_2

is mainly influenced by the longest correlation time, whereas T_1 and T_1 's frequency dependence determined on commercial spectrometers is sensitive to relatively rapid motions with τ_c in the range $10^{-8} - 10^{-10}$ sec.

To obtain information about the slower motions of hydrated water, the measurement of the proton longitudinal relaxation time in the rotating frame ($T_{1\rho}$) for water in agarose gels were undertaken in paper 6. The expression for $T_{1\rho}$ employing a weak collision approach is²³:

$$\frac{1}{T_{1\rho}} = C \left(\frac{1.5 \tau_c}{1 + 4\omega_1^2 \tau_c^2} + \frac{2.5 \tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{\tau_c}{1 + 4\omega_0^2 \tau_c^2} \right) \quad [10]$$

where ω_1 is the angular frequency of nuclear spins in the rotating frame. Since $\omega_1 \ll \omega_0$, the dependence of $T_{1\rho}$ on ω_1 is sensitive to slower relaxation producing interactions possessing correlation times longer than about 10^{-7} sec.

All the relaxation processes for ^1H in agarose gels were found to be exponential, thus indicating fast exchange of protons among different states. However, it can not be decided from NMR measurements with accuracy better than several percent whether all water protons contribute to the observed NMR signal.

The ω_1 dependence of $1/T_{1\rho}$ for protons in agarose gels exhibited similar behavior for different samples. The values of $1/T_{1\rho}$ approached $1/T_2$ as $\omega_1 \rightarrow 0$, decreased continuously with increasing ω_1 and reached limiting values at high ω_1 . The analysis of the dependence of $T_{1\rho}$ on ω_1 and of T_1 and T_2 measurements led to the following conclusions:

- A small fraction of protons in agarose-water systems is characterized by the correlation time $4-5 \times 10^{-6}$ sec at room temperature and dominates the observed T_2 . This fraction has been ascribed to firmly bound water and is of the

order of 0.003 for a 7% gel (i.e. $\simeq 0.75$ molecules H_2O per agarose monomer).

- The motion of the majority of the exchangeable protons is not described by a singel correlation time. The distribution of τ_c from $\simeq 10^{-8}$ sec to the values for ordinary water is presumably characteristic of this fraction. Higher mobilities are the most common.
- As the temperature is increased, part of the firmly bound water is successively released or at least becomes described by much shorter correlation times. The gel-to-sol transition should consequently be accompanied by the disappearance of this slow motion.

A similar study of the dispersion of $T_{1\rho}$ for protons in biological tissues was recently reported²⁴. However, the authors identified the slowest τ_c with the exchange time for protons between water molecules in the environments characterized by a large difference in the chemical shifts. It is interesting to note that the τ_c 's determined for different tissues in this study are very close to the values obtained in paper 6. The results of paper 6 indicate the presence of rapid exchange for water protons in agarose gels in accordance with case a) discussed above. The existence of inter- and intracellular media in tissues, as well as the greater heterogeneity and complexity of these systems, may render NMR observations of water in tissues not directly comparable with the results for simpler systems.

III. Diffusion permeability of membranes.

The transport of neutral molecules and ions across biological and model membranes is a subject of extensive studies on the function of biological membranes. Radioactive tracers studies

and osmotic methods are of the greatest use in measurements of membrane permeabilities. In some cases another methods have been employed, such as flame emission spectroscopy for studies of membrane permeabilities to alkaline metal ions.

NMR has not been used to a great extent in this field, mainly due to its low sensitivity. Measurements of rapid diffusion across membranes (e.g. water permeability) has, nevertheless, been performed using NMR since such rapid exchange rates are not easily measured by radioactive tracers²⁵.

Relaxation methods.

Up to now, the NMR investigations of membrane permeabilities reported in the literature have been concerned with relaxation measurements. The measurements of water permeability of nerve cell membranes²⁶ and of water exchange between human erythrocytes and blood plasma²⁷ have been achieved using ^1H NMR relaxation. These methods are based on the same principle - the addition of paramagnetic ions to the extracellular medium. Paramagnetic ions (e.g. Mn^{2+} , Gd^{3+}) significantly reduce the values of T_2 for water protons exchanging rapidly between bulk solution and the hydration spheres of these ions. Furthermore, during the time necessary for the measurement, the paramagnetic reagents usually do not penetrate membranes. When water diffuses across a membrane, it exchanges between one medium where the ^1H relaxation is slow and another medium where the ^1H relaxation is fast. The whole process can be treated as an exchange between two sites characterized by different relaxation times. If the addition of paramagnetic ions is large enough to make the T_2 of extracellular water protons much shorter than the mean lifetime of water in intracellular space (τ_{intr}), the transverse relaxation of intracellular water protons will be given by²⁸:

$$\frac{1}{T_2} = \frac{1}{T_{2,\text{intr}}} + \frac{1}{\tau_{\text{intr}}} \quad [11]$$

where $T_{2,\text{intr}}$ is the relaxation time of intracellular water protons in the absence of exchange. If $\tau_{\text{intr}} \ll T_{2,\text{intr}}$ the observed T_2 for intracellular water protons is equal to τ_{intr} .

In paper 7 NMR relaxation measurements were employed to determine water permeability of dipalmitoyl lecithin (DPL) vesicles. Vesicles are generally assumed to be spheroidal, single shell sacs of fairly uniform size (approx. 250 Å diameter for egg yolk lecithin) and can be produced from the coarse phospholipid dispersions in water by exposure to ultrasonic radiation²⁹. Each vesicle contains an aqueous space enclosed by a lipid bilayer. This model with known surface area and geometry is particularly suited for measurements of lipid membrane permeabilities. The above described relaxation method for determining water exchange rates could not, however, be applied to vesicular systems since the lifetime of water inside vesicles is very short and the addition of a large amount of paramagnetic ions decreases the stability of the lipid suspension. Therefore, vesicles were produced by sonication of the DPL suspension in water containing added Mn^{2+} , followed by an exchange of Mn^{2+} by Na^+ in the external solution. Since the fraction of intravesicular water is small and manganese ions were inserted inside the vesicles, the NMR relaxation rates observed for a majority of water protons in the systems studied were given by Eq. 5. The mean lifetime of water inside DPL vesicles was thus calculated in the temperature range 16-57°C from the experimental T_1/T_2 ratio and from the dependence of T_2 on Mn^{2+} concentration. The measurements were performed with small sample volumes, and values of τ_B as short as 0.2 msec could be determined. The permeability of DPL vesicles

to water was calculated to be 1.7×10^{-4} cm/sec at 20°C and about 17×10^{-4} cm/sec at 44°C . This diffusional process was described by an activation energy of 15 ± 1 kcal/mole in the temperature range $16\text{--}35^{\circ}\text{C}$. The temperature dependence of τ_B exhibited a slight minimum at temperatures close to the transition temperature of DPL. This finding is interesting since a similar phenomenon, although more pronounced, was observed for the permeability of DPL to Na^+ ³⁰.

NMR relaxation methods for the measurement of water diffusion across membranes are simple, rapid and require small sample volumes. The temperature dependence of the water exchange rates can be measured on one sample. These methods have, however, a drawback in that the addition of paramagnetic ions could render the observed exchange rates not directly comparable to those of non-doped samples. From that point of view the diffusion methods for the measurement of membrane permeability are more advantageous.

Diffusion methods.

The diffusion methods have been described in paper 8 for rapidly penetrating species and in paper 9 for slowly penetrating species. In both cases NMR diffusion techniques were used in systems exhibiting restricted diffusion on the NMR time scale.

The direct NMR measurement of self-diffusion is based on studies of spin-echoes in the presence of a magnetic-field gradient. For a nuclear spin of gyromagnetic ratio γ , the factor R by which diffusion will attenuate the spin-echo amplitude is given, for the pulsed-gradient experiment, by ^{31,32}:

$$R = \exp \left[-\gamma^2 \delta^2 g^2 \left(\Delta - \frac{1}{3} \delta \right) D \right] \quad [12]$$

where δ is the duration of each gradient pulse of magnitude g , Δ is the spacing between gradient pulses (i.e. the time over which the diffusion is studied) and D is the self-diffusion coefficient. Thus, for a given set of experimental parameters the echo amplitude decreases with increasing D .

Eq. 12 applies for a nuclear spin undergoing a free, three-dimensional diffusion described by Fick's law. This equation is not valid for biological systems characterized by dimensions which restrict diffusion of molecules and ions. A molecule moving about inside a cell is free to diffuse until it reaches the cell membrane. If the molecule can not leave the cell it will be turned back into the bulk fluid inside the cell. The translation diffusion of such a molecule will be restricted by the cell membrane if the diffusion is studied over times much longer than the time the molecule requires to traverse the cell cavity. Under these conditions the echo amplitude decreases with diffusion time Δ and reaches an asymptote for large Δ . This phenomenon can be illustrated by Eq. 12 if D is considered as a function of Δ , the function decreasing with Δ for diffusion times large compared with the time the molecule requires to traverse the cell cavity. Restricted diffusion has been observed in several systems³³⁻³⁵, and expressions for echo attenuation derived for corresponding model systems. For a molecule diffusing inside a spherical cavity, the asymptotic value of R is given by³³:

$$R_{\infty} = \frac{9}{C^4} \left(\frac{\sin C}{C} - \cos C \right)^2 \quad [13]$$

$$\text{with } C = \gamma \delta g a$$

where a is the cavity radius.

In many biological systems such as blood the diffusion of molecules and ions inside cells is restricted in the manner

described above, whereas this diffusion may be considered practically free in the extracellular medium. For long diffusion times Δ there will be a difference in the apparent diffusion coefficients between diffusing species in the external and internal medium. This difference arises in vivo and may be employed for measurements of membrane permeability by NMR. The principle of the measurement is different for slowly and rapidly penetrating species.

In paper 8 water diffusion permeability of human erythrocytes was studied using the NMR diffusion technique. The expressions describing the effect of molecular diffusion on spin-echo amplitude for the case of an exchange between two sites characterized by different diffusion coefficients were used^{36,37}. These expressions, generally quite intractable, may be simplified when diffusion at one of the sites is restricted. For a long Δ the effect of diffusion at this site can be neglected. The observed dependence of R on diffusion time Δ for water protons in human blood was non-exponential and the diffusion coefficient obtained from the latter part of this function could be related to the mean lifetime of water within the cells. In this manner the exchange time of 17 msec at 25°C was found for water in human erythrocytes, in good agreement with literature data³⁸. It was also found that sulfhydryl reagents, known to inhibit water osmotic permeability, significantly reduced water diffusion across the red cell membrane.

Diffusion measurements described in paper 8 have the advantage of studying exchange rates in vivo and may be used for quite a broad range of exchange times, providing the exchange time is shorter than the transverse relaxation time of the nuclear species studied.

In paper 9 the NMR diffusion technique was used to measure membrane permeability to slowly penetrating species. The measurements were performed on Li^+ in human blood. In contrast to the preceding article which concerned exchange diffusion permeability (i.e. permeability measurements in the absence of concentration gradient), paper 9 deals with investigations of Li^+ influx into human erythrocytes.

For a slow exchange of Li^+ between the extracellular (A) and intracellular (B) medium the attenuation of the ^7Li spin-echo amplitude is given by:

$$R = P_A \exp (-KD_A) + P_B \exp (-KD_B) \quad [14]$$

where $K = \gamma^2 \delta^2 g^2 (\Delta - \delta/3)$, D_A and D_B are the diffusion coefficients for Li^+ in medium A and B, P_A and P_B are the fractions of Li^+ in medium A and B, respectively. Since Li^+ diffusion inside red blood cells is restricted, Eq. 14 for measurements performed with large Δ values reads:

$$R = P_A \exp (-KD_A) + P_B R_\infty \quad [15]$$

where R_∞ is independent of Δ and D_B and is given by Eq. 13 for spherical cells. Choosing suitable experimental conditions (the value of K), the first term in Eq. 15 can be made negligible. Thus, the contribution of lithium ions in the extracellular solution to the echo amplitude can be eliminated, even when P_A is much greater than P_B . The residual echo arises from Li^+ located inside the cells and its amplitude is proportional to the intracellular concentration of Li^+ . Under these conditions, the influx or efflux of molecules and ions may be measured from changes in the residual echo amplitude with time. When K was held constant the influx of Li^+ into human erythrocytes studied in paper 9 was accompanied by the appearance and a subsequent continuous increase of the ^7Li spin-echo. The process was found to be expo-

nential, with a half time of approximately 7.5 hr at 35°C. Also the measurements of R as a function of Δ confirmed the presence of restricted diffusion of Li^+ in blood samples.

The method described in paper 9 needs minimal sample manipulations. Using the Fourier transform diffusion technique³⁹, this NMR method is particularly suited for measurements of membrane permeability to several diffusing species simultaneously. The differentiation between extra- and intracellular spaces can be achieved without disturbing the system mechanically or chemically. This might be of importance in studies of intracellular processes.

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